This is the accepted manuscript of the article, which has been published in *Journal of Biomedical Materials Research Part A*. 2017, 105(2), 356-362. http://dx.doi.org/10.1002/jbm.a.35920

Mixture of PLA-PEG and Biotinylated Albumin enables Immobilization of Avidins on Electrospun Fibers

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Abstract

The application of nanotechnology in biomedical field has enormous potential in basic and applied research. Micro or nanofibers produced by electrospinning technique offer excellent properties because of large specific surface area, high porosity and ability to incorporate functional additives. Here we embedded biotinylated bovine serum albumin into polylactic acid (PLA)-polyethylene glycol (PEG) fibers, which enabled specific immobilization of fluorescently labelled avidin. An alkaline phosphatase enzyme was immobilized via biotin-streptavidin interaction on the hybrid nanofibers, demonstrating the suitability of the material for biosensing applications. These functional nanofibers provide a promising platform for development of biosensors and other biofunctional materials utilizing avidin-biotin as a generic and robust immobilization method.

Keywords: Electrospinning, Polylactic acid (PLA); Polyethylene glycol (PEG); Biotinylated BSA, Avidin, Functional fibers

Introduction

Electrospinning is a straight-forward approach for creating highly functional continuous one-dimensional nanofibers (1), (2), (3), (4), (5). Electrospun polymeric micro- and nanofibers provide unique properties, such as inherently high surface-area to-volume ratio, high interconnectivity, porous structure, and high flexibility in surface functionality. Fibers with complex architecture such as randomly oriented, aligned fibers, core-shell fibers, hollow fibers, porous fibers, side-by-side structures can be easily produced with this technique (6), (7). These fibers have desirable properties for advanced biomedical applications such as biosensors, controlled drug delivery, tissue engineering, enzyme carriers and much more (8), (9), (10), (11), (12). During electrospinning, one of the important challenges is the control of the morphology and the geometry of the nanofibers. All in all, nanofibers open the door to dramatically improve the performance of existing technologies/devices.

When fibers are considered for biosensing applications, there are three important factors to be considered (13), (14): (1) hydrophilicity; (2) surface functionalities and (3) biocompatibility. The hydrophilicity of the fibers must be balanced to avoid aqueous degradation. The fiber surface must be functional which provides the ability to interact with bioactive surface. And it must be biocompatible to avoid injurious effects on the living body. For this purpose, we have chosen bio-absorbable, bio-degradable, bio-based polymer; polylactic acid (PLA), as a base material for functionalization using avidin-biotin interaction. Avidin-biotin was selected due to its high specificity, greater availability of the suitable functionalization tools (biotinylated antibodies, nucleic acids, nanoparticles, etc.) and also because genetically engineered avidins (15) makes it possible to meet more specific needs in biofunctionalization. PLA represents the front-runner bio-based polymer due to its attractive features such as renewability, biocompatibility and high thermo-mechanical properties (16), (17), (18), which brings a large set of applications from biosensor to packaging. However, there are some

shortcomings to be addressed, especially for biosensor applications, high hydrophobicity being one of the important challenges (19). A range of modifications, including chemical, physical, biological and nanotechnology techniques are therefore proposed to improve the characteristics of PLA (20). A simple method is physical blending with hydrophilic polymer such as polyethylene glycol (PEG) (21), (22). The addition of PEG to PLA is mainly to decrease the hydrophobicity, which improves the wettability of the final fibers (23). PEG is chosen as plasticizer which increases the free volume of the polymer/plasticizer system by augmenting the space between polymer chains and thus causing them to spread apart. During the electrospinning process, PEG in copolymers of PLA-PEG has been found to become segregated, leading to a fiber with hydrophilic PEG at the surface (24). This results in fibers with higher homogeneity and wettability as compared to fibers made of PLA alone.

Biotin (244.3 g/mol) is chemically inert, water-soluble and optically inactive B-vitamin, also called vitamin H. Avidin is a homotetrameric protein known to bind biotin with high affinity ($K_d \sim 10^{-16}$ M). This protein-ligand pair has been widely utilized in applications ranging from biosensors to targeting of gene therapy. One promising application for avidin-biotin is a cancer therapy method called pretargeting radioimmunotherapy (25). In addition to avidin isolated from chicken eggs, there are improved genetically modified versions of avidin available. For example, so called chimeric avidin has been found thermostable, resistant for proteolysis and suitable for biofunctionalization of various materials, while retaining high affinity ligand binding (26). Chimeric avidin remains stable during long periods of storage and provides cost-effective manufacturing of biochips and biosensors. Another example of engineered avidins is switchavidin, which can be removed from biotinylated surfaces with mild treatment, enabling re-use of the biotinylated device (27). Due to four biotin-binding sites per avidin, this protein-ligand pair used as a signal-amplifier to sandwich molecules responsible for specific binding and detection.

Binding between biotin and avidin is very tight, and several studies have demonstrated that usually sufficient conditions for protein denaturation are failing to weaken the binding between avidin-biotin complexes (28). It is also possible to independently control individual binding sites and alter their affinity for the ligands (29). Once the avidin-biotin complex is formed, it is unaffected by wide extremes of pH (between 2 and 13), high temperature (melting point around 120°C), organic solvents, or other denaturing agents. Avidin retains its ability to bind to biotin at room temperature in the presence of common surfactant such as SDS, SDBS and Triton X-100. This protein-ligand pair has proved ideal for immobilization of single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA). Figure 1 shows a schematic diagram of avidin immobilization on biotinylated fibers and their blocking with free biotin.

In the present investigation, we successfully synthesized biotinylated bovine serum albumin (BSA) embedded PLA-PEG as blend fiber membrane. These blend fiber membranes were able to immobilize chimeric avidin. In contrast, excess of free biotin blocked the binding sites of avidin and prevented binding to fibers, confirming the specificity of the interaction. Furthermore, fibers produced without biotinylated BSA showed no binding of avidin. We also used enzymatic assay to demonstrate the accessibility of the biotin groups on the nanofibers for suitable attachment of streptavidin-functionalized alkaline phosphate enzyme.

Materials and methods

All chemicals are of analytical grade and used without prior treatment. Poly(lactic acid) 6202 (PLA) was provided by Prof. Endres, Institute for Bioplastics and Biocomposites (IFBB) Hannover, Germany. Biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester (Biotin-X-X-NHS) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. Poly(ethylene glycol) (PEG) 1500 was purchased from abcr, and chloroform (CLF) 99.8% (UV/IR-Grade) from Roth. Deionized water was used throughout the process.

Chemical coupling of biotinylated BSA

Biotinylated BSA was produced by chemically coupling biotin on the amino groups of bovine serum albumin. To prepare this, 2.95 g of BSA was dissolved in 28.5 ml of 50 mM sodium phosphate buffer containing 100 mM NaCl (pH 7). In the separate flask, 25 mg of biotin-X-X-NHS was dissolved in 1 ml of DMF. The biotin-X-X-NHS solution was directly added to the BSA solution. The mixture was incubated one hour at room temperature (RT) on a shaker. Biotinylated BSA was then dialyzed against 50 mM sodium phosphate, 100 mM NaCl (pH 7) o/n and stored -20°C in aliquots.

Synthesis of PLA, PLA-PEG and biotinylated PLA-PEG nanofibers

Three homogeneous electrospinning solution sets were prepared, in one set, 8 wt% PLA are dissolved in chloroform ($CHCl_3$) solvent and stirred for 10 hours to obtain (a uniform) viscous solution. Chloroform was chosen as solvent because it improves the dispersion of biotinylated BSA (30). In the second set, PEG (1:1 wt%) added to the prepared PLA solution. The addition of PEG was mainly to decrease the hydrophobicity and increase the stability and flexibility of the fibers (23). And in the third set, biotinylated BSA (0.01 wt%) was slowly dispersed to the prepared PLA-PEG blend solution and ultrasonicated (Bandelin Electronic) for 10 minutes in 1 minute interval to get a clear dispersion. All the three sets of sample, i.e. (1) 8 wt% PLA, (2) 8-8 wt% PLA-PEG blend and (3) 8-8-0.01 wt% PLA-PEG-biotinylated BSA blend (referred later as "biotinylated PLA-PEG") was separately filled in a 5 mL plastic syringe equipped with a blunt end stainless steel needle having a size of 0.80×22 mm^2 (21×7/8 G''). During electrospinning at room temperature, a positive high voltage was maintained between needle (as a positive terminal) and stainless steel flat surface covered with aluminum foil (as negative terminal). Under this applied electrostatic field, the polymeric hemi-sphere droplet surface gets elongated to form a "Taylor cone" and when it reaches the critical point, the repulsive force overcomes the surface tension of the polymeric

solution. Hence, the charged jet was emitted to from the end of Taylor cone and converted into a web of fibrous material which is collected on the grounded substrate (31). This electrospun membrane was then peeled off from the collector and kept for drying at room temperature to remove the residual solvent. Table 1 shows the parameters used for the electrospinning experiment.

Material characterization

Scanning electron microscopy (SEM)

The surface morphology and diameter of the prepared fibers were observed by scanning electron microscope (SEM) Zeiss Leo VP 1455. Three samples were prepared; (1) PLA fibrous membrane, (2) PLA-PEG blend fibrous membrane and (3) biotinylated PLA-PEG fibrous membrane. A thin layer of gold/platinum was sputtered before taking SEM image to make the fiber conductive. The mean fiber diameters were analysed using digimizer software (Digimizer, version 4.5), with an error bar (standard deviation).

Determination of the availability of the biotin using fluorescence microscopy

For fluorescent microscopy, individual PLA-PEG blend fibers and PLA-PEG blend fibers containing biotinylated BSA were used to detect the biotin molecules on the fiber surface. This blend fiber membranes were shred into pieces by using tweezers and immersed into 0.05% PBS-Tween (pH 7.4). First, the samples were incubated for 30 min in 0.5% BSA in PBS-Tween to minimize non-specific binding. For fluorescent staining, the samples were incubated for 120 min in 150 nM of neutralized chimeric avidin (26) labeled with amino-reactive AlexaFluor-488 (Life Technologies). For negative samples, 150 μM biotin was added to the avidin solution 10 min before the sample incubation. All samples were washed with three times (5 min + 30 min + 30 min) with PBS-Tween and mounted between coverslips by using Prolong Gold mounting media (Life Technologies).

Stained samples were imaged by using Zeiss LSM780 laser scanning confocal microscope equipped with 63x / NA 1.4 oil immersion objective. For each field, a Z-stack of 50 µm was imaged at 2 µm intervals. The imaged field size was 224 µm*224 µm. Bright field image was captured by using a transmitted light PMT detector. All imaging parameters were kept constant for all samples.

Demonstration of enzymatic assay on biotinylated nanofibers

To demonstrate the suitability of the biotinylated fibers for enzymatic assays, streptavidin-alkaline phosphatase was used as a probe. For this, three different fiber membranes of a 0.5 mg piece (app. 3 mm x 3 mm) were cut; 1) PLA fibers 2) PLA-PEG blends fibers 3) biotinylated PLA-PEG blend fibers. All the samples were analyzed in duplicate.

For convenient sample handling, the protocol was performed in 96-well microplate. The samples were first immersed in PBS (10 mM phosphate buffer, 140 mM NaCl, 2.7 mM KCl pH 7.4) containing 1 mg/ml BSA for 1 hour and then washed three times with PBS. Streptavidin-alkaline phosphatase (SA-AP) solution were prepared by diluting SA-AP (Roche prod. no. 11 093 266 910) 1:3000 in PBS containing 0.1 mg/ml BSA. The negative control solution was prepared by including 17 µg/ml biotin (Sigma B-4501) to the diluted SA-AP, followed by 1 h incubation. The SA-AP solutions were applied on the fiber membrane samples for 1 hour, followed by three washes with PBS. After moving the samples to new microwells, the samples were again washed three times with PBS. A substrate solution for AP was applied to the samples. After 30 min incubation, the substrate solution was transferred to empty wells and the absorbance was measured at 405 nm. All the measurement was performed at room temperature (RT). A substrate solution in empty microwell was used as a reference sample.

Results

Fiber morphology studies by scanning electron microscopy (SEM)

SEM image shows that prepared fibers were randomly orientated, porous, beadles, smooth and interconnected. Figure 2a revealed that electrospun PLA fiber membrane was porous and cylindrical (32), this could happen because of rapid evaporation of solvent ($CHCl_3$) during electrospinning. The mean diameter of the fibers was 5.9 ± 0.3 µm. Figure 2b shows PLA-PEG blend fibers with diameter 2.9 ± 0.3 µm. Figure 2c shows biotinylated BSA dispersed PLA-PEG blend fibers which reveal that the fibers were still cylindrical and smooth with a mean diameter 1.3 ± 0.4 µm. We observed that the addition of biotin-BSA to the spinning dope did not affect the final morphology Figure 2d shows mean fiber diameter for various mixtures used.

Enzymatic detection of the biotin

As biotinylated BSA molecules were distributed throughout the fiber surface, enzymatic assays were performed to demonstrate the biotin availability at the surface of fibers. The method consisted of adding 0.5 mg (app. 3 mm x 3 mm) fiber membrane into a streptavidin-alkaline phosphatase (SA-AP) solution. Binding of the enzyme was detected by measuring the immobilized enzymatic activity with the chromogenic substrate at 405 nm. When a fiber membrane containing biotin was added to the SA-AP solution, clear enzymatic activity was observed due to fiber-immobilized enzymes. We also observed that no SA-AP immobilization on PLA and PLA-PEG fibers took place. This confirms that the presence of biotin at the fiber surface caused the immobilization of the enzyme.

Wettability is one of important parameter which influenced the enzymatic assays (13); therefore we had incorporated PEG to PLA, which makes SA-AP solution easier to penetrate. This (incorporation of PEG to PLA) also increased the surface area of the membrane from 2.1 to 5.0 m^2/g (Table 2) which improved the accessible surface area and possibly also enhanced migration of the biotin to the fiber surface.

Fibers for avidin immobilization

The biotinylated BSA incorporated PLA-PEG blends fibers were conjugated with neutralized chimeric avidin using a simple single-step binding assay. Two sets of experiments were performed. In the first set, PLA-PEG blend fibers were cut and incubated with 150 nM AlexaFluor-488 labeled neutralized chimeric avidin for 120 min at room temperature. In the second step avidin-biotin interaction was blocked with excess of free biotin. The same experiment was also performed for biotinylated PLA-PEG blend fibers.

Our result shows immobilization of chimeric avidin on biotinylated PLA-PEG blend fibers via adsorption onto the fiber surface membrane. In contrast, no immobilization of avidin molecules on PLA-PEG fibers were observed. This was expected because PLA-PEG fibrous membrane does not contain biotin. These results shed promise for the use of the material for biosensor applications. This is also studied by Frey et al. (30), (33) and Lu et al. (34).

After immobilization of avidin molecules on fiber surface (Figure 3i), the binding specificity was proven by blocking the binding by preincubation of avidin in the presence of excess of free biotin (Figure 3l). Figure 4 shows histogram analysis of the samples, including biotin binding. From this we could conclude that avidin molecules were only immobilized on biotinylated PLA-PEG fibrous membrane and no immobilization on PLA-PEG fibrous membrane was observed.

Discussion

Electrospun fibers have unique properties, as such as high aspect ratio, high surface area, high porosity, and outstanding properties (35), which make them suitable material for designing biosensors. We observed a significant decrease in fiber diameter when PEG copolymer were added to PLA solution. This was expected because PEG works as a plasticizer which create points of attraction with the polymer chains and leave an unattached portion. Indeed, this increase in free volume of the system by augmenting the space between polymer chains in spreading them apart [12]. It is considered that the addition of the flexible

PEG chains decrease the solution electrical conductivity, viscosity and surface tension of the spinning solution, results in a decreased fiber diameter (36).

We also found that the biotinylated electrospun PLA-PEG fibers could be functionalized with neutralized chimeric avidin (also called as nChiAvd) and this can be blocked by treating with an excess of free biotin. Functionalization via genetically engineered avidins leads to new and improved properties when compared to conventional micro or nanofibers. The observed phase separation of biotin-BSA towards the fiber surface membrane were happened most probably because of applied electric voltage during fiber formation process (33) and also due to lack of entropy during polymer mixing (37), which results in more biotin at the surface of fiber membrane. The resulting fibrous membrane shows fast immobilization of avidin molecules, which open the door for paper based biosensors (38), (39), (40). Recently, avidin enabling release from biotinylated materials has been described, offering a potential way to develop regenerable biosensors (27), (41), (42).

In conclusion, biotinylated BSA surface functionalized PLA-PEG blend fibers were successfully prepared by electrospinning technique. PLA fibers showed porous, beadless and interconnected structure. PLA-PEG blend fibers showed a decreased fiber diameter with smooth morphology. Biotinylated PLA-PEG blend fibers also showed uniform morphology. In addition, biotinylated BSA at the fiber surface was used to couple fluorescent avidin molecules. This biotin-avidin binding on fiber surface could be blocked by excess of free biotin. This functional hydrophilic fiber shows potential application for biosensor applications.

Acknowledgements

The authors gratefully thank Dr. Jenni Leppiniemi for preparation of biotinylated BSA. Folke Dencker is acknowledged for his support with the scanning electron microscopy (SEM)

and Marc Krey for Brunauer Emmett Teller (BET) analysis. The work was supported by grants from Hannover School for Nanotechnology, Germany and from Academy of Finland.

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Figure legends

Figure 1. Immobilization of avidin on biotinylated fiber. (a) Avidin immobilized on biotinylated fiber. (b) Free ligand-binding sites of avidin are blocked by an excess of free biotin.

Figure 2. Scanning electron micrographs for electrospun (a) PLA fibers (b) PLA-PEG blend fibers (c) Biotinylated PLA-PEG blend fibers (d) and their average diameter distribution.

Figure 3 (a) Shows a fluorescent confocal microscopy image of the PLA-PEG fibers. (b) The same fibers were immersed in fluorescently labelled avidin solution and fluorescence at 488 nm was measured. (c) PLAPEG fibers merged with fluorescently labelled avidin, which clearly indicate no immobilization of avidin on the PLA-PEG fibers because the biotin binding sites in avidin were not present. (d-f) The non avidin immobilized PLA-PEG fibers were treated with excess of biotin. There were no blocking occurred due to unavailability of avidin molecules. (g-l) The same analysis as in (a-f) was conducted for PLA-PEG fibers containing biotinylated BSA, which clearly indicate immobilization of avidin on fibers. (j-l) indicates the same analysis, where the immobilized avidins are blocked with excess of free biotin.

Figure 4. Fluorescence histogram analysis of avidin immobilization on biotinylated PLA-PEG blend fiber (dark blue). Biotinylated PLA-PEG blend fibers treated with avidin blocked with excess of free biotin (biotin-blocked negative control; light blue). PLA-PEG blend fibers show no binding (negative control; red). PLA-PEG blend fibers without biotin functionalization treated with biotin-blocked avidin studied as a comparison (pink).

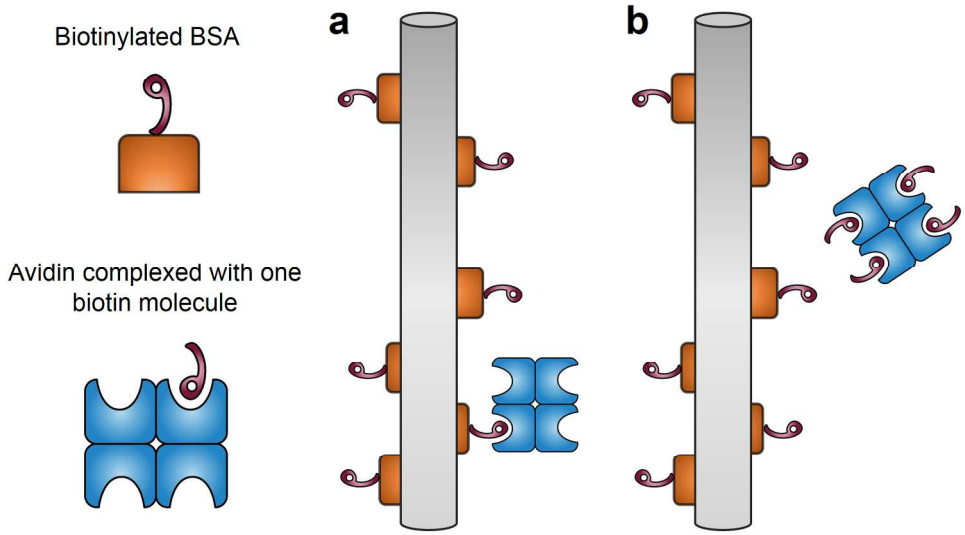

Figure 1. Immobilization of avidin on biotinylated fiber. (a) Avidin immobilized on biotinylated fiber. (b) Free ligand-binding sites of avidin are blocked by an excess of free biotin.

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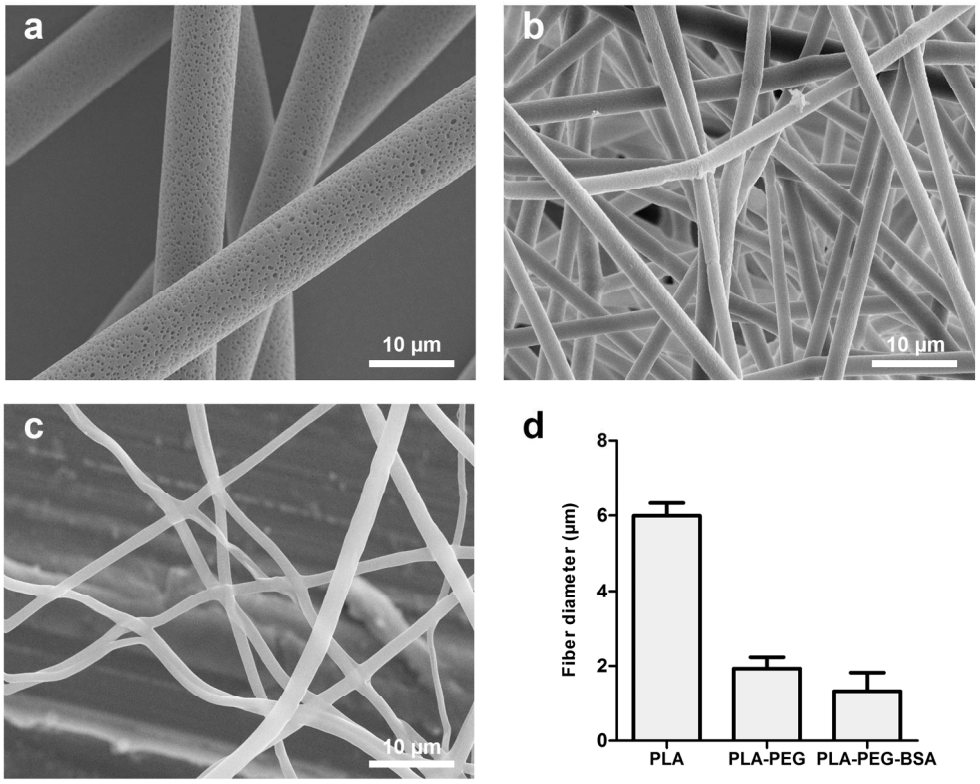

Figure 2. Scanning electron micrographs for electrospun (a) PLA fibers (b) PLA-PEG blend fibers (c) Biotinylated PLA-PEG blend fibers (d) and their average diameter distribution.

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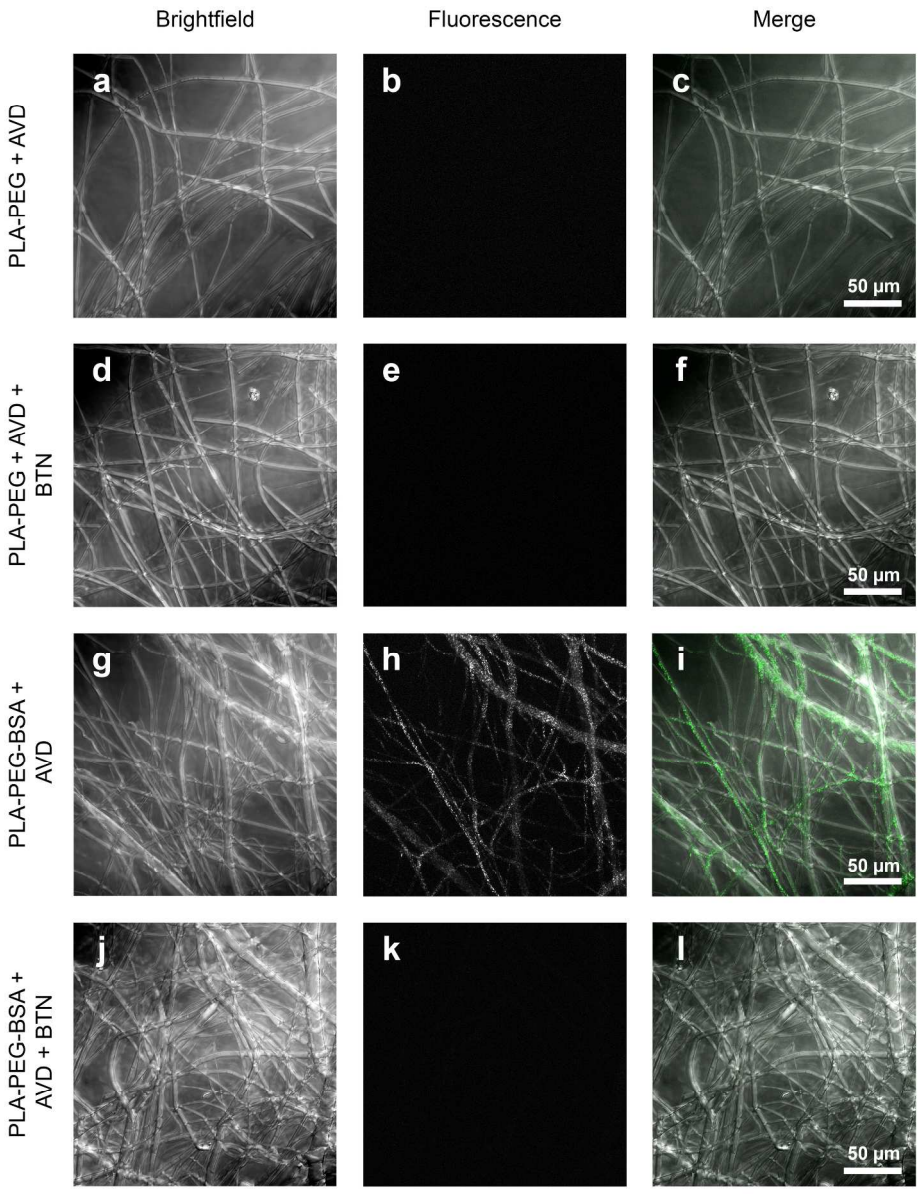

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227x293mm (300 x 300 DPI)

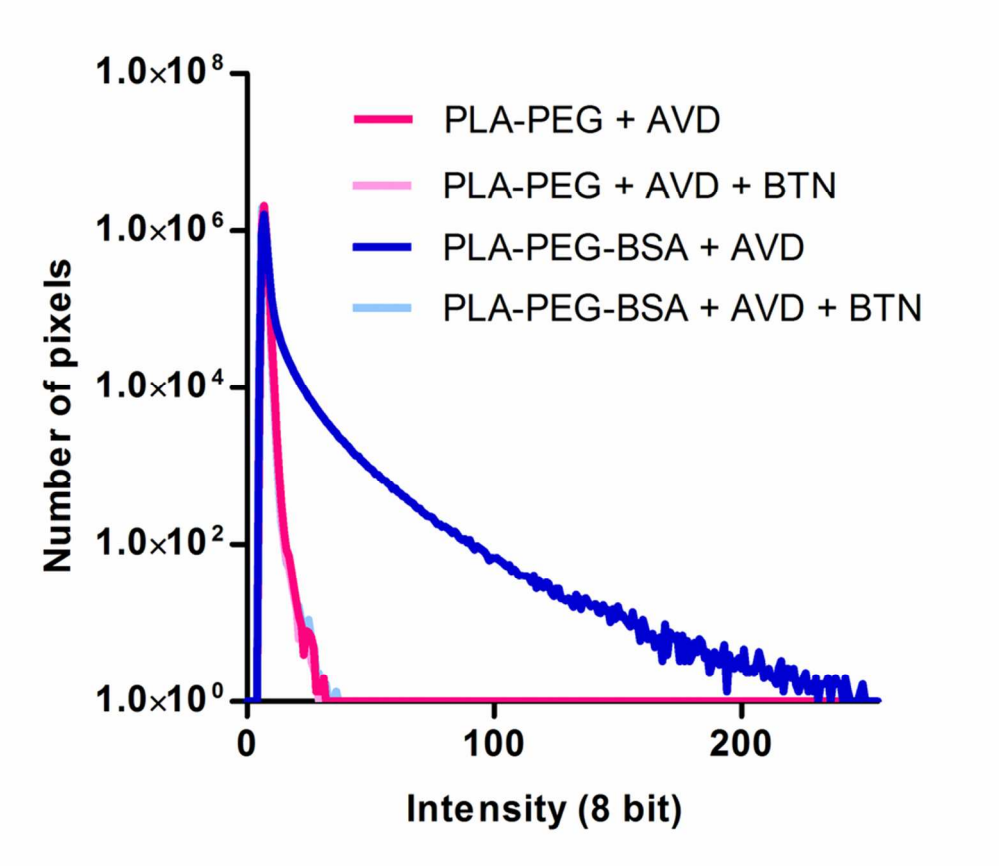

Figure 4. Fluorescence histogram analysis of avidin immobilization on biotinylated PLA-PEG blend fiber (dark blue). Biotinylated PLA-PEG blend fibers treated with avidin blocked with excess of free biotin (biotin-blocked negative control; light blue). PLA-PEG blend fibers show no binding (negative control; red). PLA-PEG blend fibers without biotin functionalization treated with biotin-blocked avidin studied as a comparison (pink).

82x72mm (300 x 300 DPI)

Sample	Concentration [%Wt]	Flow rate [mL h^{-1}]	Voltage [kV]	Height [cm]	Mean Diameter [μm]
PLA	8	1	12	13	5.98 ± 0.33
PLA-PEG	8-8	1	12	15	2.92 ± 0.30
Biotinylated PLA-PEG	8-8-0.01	1	18	13	1.15 ± 0.28

Table 1: Summary of electrospinning conditions.

Table 2: The BET surface area of PLA and PLA-PEG blend fibrous membrane

Sample	**Surface Area of the Fiber (m^2/g)**
PLA	2.1
PLA-PEG	5.0